

The Catabolite Control Protein CcpA Controls Ammonium Assimilation in *Bacillus subtilis*

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Abstract

Carbon catabolite repression of several catabolic operons in *Bacillus subtilis* is mediated by the repressor CcpA. An inactivation of the *ccpA* gene has two distinct phenotypes: (i) catabolite repression of catabolic operons is lost and (ii) the growth of bacteria on minimal medium is severely impaired. We have analyzed the physiological properties of a *ccpA* mutant strain and show that the *ccpA* mutation does not affect sugar transport. We have isolated extragenic suppressors of *ccpA* that suppress the growth defect (*sgd* mutants). Catabolite repression of β -xylosidase synthesis was, however, not restored suggesting that the suppressor mutations allow differentiation between the phenotypes of the *ccpA* mutant. A close inspection of the growth requirements of the *ccpA* mutant revealed the inability of the mutant to utilize inorganic ammonium as a single source of nitrogen. An intact *ccpA* gene was found to be required for expression of the *gltAB* operon encoding glutamate synthase. This enzyme is necessary for the assimilation of ammonium. In a *sgd* mutant, *gltAB* operon expression was no longer dependent on *ccpA*, suggesting that the poor expression of the *gltAB* operon is involved in the growth defect of the *ccpA* mutant.

Introduction

Bacillus subtilis is able to utilize several sugars as carbon sources. In the presence of more than one sugar the bacteria are able to preferentially use the one allowing the fastest growth. Three proteins are central to this process in *B. subtilis*, referred to as carbon catabolite repression: the catabolite control protein CcpA, the HPr protein of the

phosphoenolpyruvate:sugar phosphotransferase system (PTS) and a protein similar to HPr but not endowed with enzymatic activity, Crh (Henkin *et al.*, 1991; Deutscher *et al.*, 1995; Galinier *et al.*, 1997). CcpA is a member of the GalR-LacI family of transcriptional regulators. In the presence of glucose and other glycolytically metabolizable carbon sources it binds to catabolite responsive elements (*cre*) in front of numerous catabolic genes and operons thus preventing their expression (for review see Hueck and Hillen, 1995). In addition, CcpA can act as an activator of gene expression in the presence of glucose as in the case of *ackA* encoding acetate kinase (Grundy *et al.*, 1993). The *ccpA* genes have recently been cloned from *B. megaterium*, *Staphylococcus xylosus*, *Lactobacillus pentosus* and *L. casei*, and their involvement in carbon catabolite repression was demonstrated (Hueck *et al.*, 1995; Egeter and Brückner, 1996; Lokman *et al.*, 1997; Monedero *et al.*, 1997). Thus, catabolite repression exerted by CcpA seems to be a common mechanism in Gram-positive bacteria. A different mechanism of catabolite regulation is operative in the absence of repressing sugars. Under these conditions HPr phosphorylates positively acting transcriptional regulators containing a PTS regulation domain (PRD) thus stimulating their activity (Deutscher *et al.*, 1997; Stülke *et al.*, 1998).

The signal for the presence of repressing sugars is generated during glycolysis. CcpA from *B. subtilis* and *B. megaterium* binds *cre* sites in the presence of glucose-6-phosphate (Gösseringer *et al.*, 1997; Miwa *et al.*, 1997). Moreover, DNA-binding of CcpA was shown to be stimulated upon binding of the HPr protein of the PTS phosphorylated at a regulatory site, Ser-46 (Fujita *et al.*, 1995; Gösseringer *et al.*, 1997; Jones *et al.*, 1997). Similarly, phosphorylation of Crh, an HPr-like protein of *B. subtilis*, at Ser-46 was proposed to result in interaction with CcpA and subsequent *cre* binding and catabolite repression (Galinier *et al.*, 1997). HPr and Crh are both phosphorylated by an ATP-dependent protein kinase in the presence of elevated concentrations of fructose-1,6-bisphosphate (Galinier *et al.*, 1998; Reizer *et al.*, 1998).

In addition to its involvement in carbon catabolite repression, the *ccpA* gene product is required for normal growth in minimal media supplemented with various sugars (Miwa *et al.*, 1994). This was observed not only for *B. subtilis* but subsequently also with *ccpA* mutants of *B. megaterium* (Hueck *et al.*, 1995). However, the growth defect is less pronounced in complex media. No or only a marginal growth defect was observed for *ccpA* mutants of *S. xylosus* and *L. pentosus*. However, no minimal media are available for these bacteria suggesting that a functional *ccpA* gene was not required under the conditions tested (Egeter and Brückner, 1996; Lokman *et al.*, 1997). It has been shown that *ccpA* mutants of *B. subtilis* require the presence of intermediates of the tricarboxylic acid cycle to grow on a minimal medium containing glucose and ammonium (Wray *et al.*, 1994). However, even in the

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Table 1. Growth of a *ccpA* Mutant on Different Carbon Sources^a

Carbon source	IS58 wild type	BGW2 <i>ccpA</i>
glucose	1.25	0.44
fructose	1.25	0.42
sucrose	1.25	0.43
glucitol	1.25	0.46
glycerol	1.25	0.43
gluconate	1.11	0.38
ribose	0.71	0.41
citrate	1.0	0.41

^a Specific growth rates (μ , h^{-1}) were measured using the compounds listed as single sources of carbon and energy in modified ASM medium (Krüger *et al.*, 1993). The cultures were inoculated with precultures grown in ASM medium.

presence of citrate and glutamate, an inhibition of growth of a *ccpA* mutant by arabinose was observed. Since this effect was not observed in the presence of ribose, growth inhibition was probably not due to catabolism via the pentose phosphate shunt but rather to some unidentified intermediates of arabinose utilization (Strauch, 1995). The growth defect of *ccpA* mutants could be circumvented by a secondary *spo0A* mutation. The Spo0A protein represses the pleiotropic regulatory gene *abrB*, and a *spo0A* mutation results in *abrB* overexpression. The faster growth observed for *spo0A* mutants has been attributed to this overproduction (Fisher *et al.*, 1994). Indeed, the *abrB* gene product was necessary for the faster growth of a *ccpA spo0A* double mutant compared to a *ccpA* strain (Strauch, 1995).

We have investigated the reason for the growth defect of the *ccpA* mutant. The data presented here indicate that glucose transport is not impaired in the *ccpA* mutant strain. Suppressor mutants (*sgd*) that affect the growth behaviour but not carbon catabolite repression were isolated allowing differentiation between the two phenotypes of the *ccpA* mutant. Surprisingly, we found the *ccpA* mutant to be similar to *glt* mutants affected in the central enzyme of ammonium assimilation, glutamate synthase, with respect to the growth deficiency. This phenotype is associated to the decrease of *gltAB* operon expression in the *ccpA* mutant strain. A *sgd* mutation which restored growth of the *ccpA* mutant strain, concomitantly restored the expression of the *gltAB* operon.

Table 2. Effect of the *ccpA* Mutation on the Expression of PTS Components in *B. subtilis*

System tested	Strain	Genotype	β -Galactosidase activity ^a	
			CSE	CSE Glc
<i>ptsHI</i> '-' <i>lacZ</i> ^b	QB5418	wild type	110	155
	GP1	<i>ccpA</i> :: <i>spc</i>	110	135
Δ LA <i>ptsG</i> '-' <i>lacZ</i> ^c	QB5448	wild type	7	100
	GP10	<i>ccpA</i> :: <i>spc</i>	8	135

^a β -Galactosidase activity is expressed in units/mg protein.

^b The *ptsHI*'-'*lacZ* transcriptional fusion (Gonzy-Tréboul *et al.*, 1989) was introduced at the *amyE* locus.

^c The Δ LA *ptsG*'-'*lacZ* translational fusion (Stülke *et al.*, 1997) was integrated at the *amyE* locus.

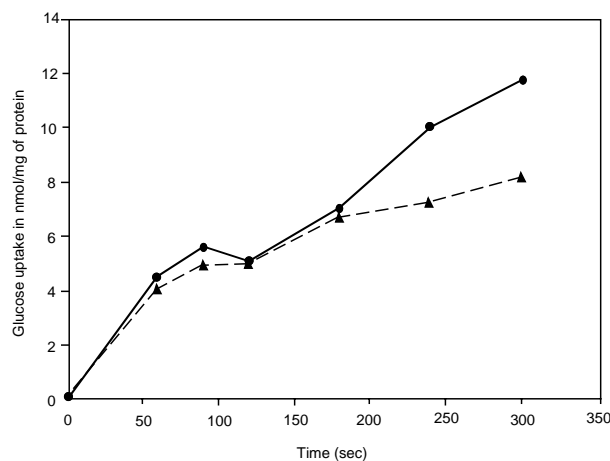


Figure 1. Transport of Glucose

The [¹⁴C] glucose uptake was measured as described in the Experimental Procedures section. The strains *B. subtilis* 168 (wild type, circles) and QB5407 (*ccpA*, triangles) were grown in CSE medium supplemented with 1% glucose.

Results

Growth of a *ccpA* Mutant Strain on Different Carbon Sources

To get insights into the nature of the growth defect of *ccpA* mutants the growth rates of *B. subtilis* IS58 and its isogenic *ccpA* counterpart BGW2 were determined on a variety of carbon sources (Table 1). Whereas the wild type strain showed the highest growth rates on glycolytically metabolizable sugars, the *ccpA* mutant exhibited slow growth on all carbon sources tested. Similar results were obtained with a *ccpA* mutant strain of *B. megaterium* (Hueck *et al.*, 1995).

Glucose Transport by a *ccpA* Mutant Strain

The low growth rate of the *ccpA* mutant on glucose and other sugars might be due to inefficient sugar transport into the cell or to a defect in the downstream catabolism. To distinguish between these possibilities, the expression and activity of the glucose-specific PTS were compared between the wild type and the *ccpA* mutant strain. The expression of the *ptsG* gene encoding EII^{Glc} is induced by glucose whereas expression of the *ptsHI* operon encoding the general enzymes of the PTS, HPr and Enzyme I, is constitutive (Gonzy-Tréboul *et al.*, 1989; Stülke *et al.*, 1997). The expression of *ptsG* and the *ptsHI* operon was assayed using fusions to the promoterless *lacZ* gene as reporter in wild type and *ccpA* mutant backgrounds. Bacteria were grown in CSE medium or in CSE medium supplemented with glucose and their β -galactosidase activities were determined (Table 2). The *ccpA* gene disruption did not affect the constitutive expression of the *ptsHI*'-'*lacZ* fusion. Expression of *ptsG* was 15-fold induced in the presence of glucose. As observed for the general PTS components, the *ccpA* mutation had no effect. These results indicated that the synthesis of the PTS proteins required for glucose transport is not altered in a *ccpA* mutant. To rule out the possibility that the glucose-specific PTS required the presence of a functional *ccpA* gene product for normal activity, the transport of glucose in *B. subtilis* 168 and QB5407 (*ccpA*::*spc*) was determined. The bacteria were

Table 3. Effect of the *sgd* Mutations on Glucose Repression of β -Xylosidase Synthesis and Growth on Glucose as Single Carbon Source

Strain	Relevant genotype	β -Xylosidase activity ^a		Generation time (min)
		CSE Xyl	CSE Xyl Glc	
168	wild type	640	4	45
QB5407	<i>ccpA</i>	1140	1125	140
QB5412	<i>ccpA sgd-1</i>	1820	555	60
QB5413	<i>ccpA sgd-2</i>	2070	520	60
QB5414	<i>ccpA sgd-3</i>	2000	690	60
QB5415	<i>ccpA sgd-5</i>	1585	590	50

^a β -Xylosidase activity is expressed in units/mg protein.

grown in CSE medium under conditions inducing *ptsG* expression (i.e. in the presence of 1% glucose). As shown in Figure 1, the *ccpA* mutation did not significantly affect glucose transport. Thus, both expression and activity of the glucose-specific PTS components did not depend on the *ccpA* gene product suggesting that some other step in catabolism may be impaired in the *ccpA* mutant.

Isolation of Suppressor Mutations for the *ccpA* Growth Defect

In the experiments described above, the cultures were inoculated using precultures grown in ASM. This medium contains citrate and glutamate which feed directly into the tricarboxylic acid cycle and might be used as carbon sources. It has been reported that TCA cycle intermediates are required by *ccpA* mutants for growth in minimal media (Wray *et al.*, 1994). We therefore tested the ability of a wild type and *ccpA* mutant strain to form colonies on C minimal plates containing glucose as a single carbon source. Under these conditions, the wild type strain *B. subtilis* 168 formed colonies within 24 h, while the isogenic *ccpA* mutant QB5407 was not able to form colonies even after 72 h. The growth defect was therefore more rigorously analyzed in liquid medium. C minimal medium containing glucose was inoculated with precultures grown in CSE medium. As observed with ASM, the *ccpA* mutant grew in this medium, albeit at a low growth rate. When this culture was then used to inoculate fresh medium containing glucose as a single carbon source, no growth occurred. These observations were exploited to select second-site suppressor mutations restoring the growth of the *ccpA* mutant in the presence of glucose as a single carbon source. Four independent mutants were selected on C plates supplemented with glucose. The presence of the original *ccpA::Tn917 spc* cassette in the suppressor mutants was verified by replacing the cassette by the *ccpA::Tn917* insertion of BGW2 conferring Erm resistance. All transformants were Erm^r and Spc^s confirming that the *spc* gene had resided in *ccpA*. The mutations were designated *sgd* (suppression of **g**rowth **d**efect). The growth rates of the *ccpA sgd* mutant strains were determined in C minimal medium with glucose as a single carbon source. As shown in Table 3, the mutants grew considerably faster than the *ccpA* strain, but growth was somewhat reduced as compared to the wild type 168. We also tested whether cultures grown in C-glucose could be used to inoculate the same medium. As described above, the *ccpA* mutant QB5407 did not grow under these conditions while all of the suppressor strains did. As our original interest in the phenotypes of the *ccpA* mutation came from the analysis

of carbon catabolite repression we wondered whether the *sgd* mutations would affect both the growth and catabolite repression phenotypes of the *ccpA* mutant. To test this possibility, the repression of β -xylosidase synthesis by glucose was compared in the wild type strain *B. subtilis* 168, the *ccpA* mutant QB5407, and its isogenic *sgd* derivatives. This repression was shown to be completely abolished in the *ccpA* mutant (Lindner *et al.*, 1994). β -Xylosidase activities were determined after growth in CSE medium in the presence of xylose as the inducer with or without glucose (1%, w/v) (Table 3). As observed previously, β -xylosidase synthesis was completely relieved from glucose repression in the *ccpA* mutant strain. Similarly, only weak repression was observed in the *ccpA sgd* double mutants (2.5 to 4-fold) compared to the 160-fold repression observed in the wild type strain. Moreover, we tested the effect of the *sgd-1* mutation on glucose repression of the *lev-* and *bgIPH*-operons in the *ccpA* background. Both operons are subject to *ccpA*-dependent catabolite repression (Martin-Verstraete *et al.*, 1995; Krüger *et al.*, 1996). As observed with β -xylosidase, repression was not restored by the *sgd-1* mutation (data not shown). Thus, we suppose that the *sgd* mutations separate the growth defect of the *ccpA* mutation from catabolite repression deficiency.

Analysis of the Growth Requirements of the *ccpA* Mutant Strain

The observation that intermediates present in CSE and ASM allowed growth of the *ccpA* mutant led us to investigate the growth requirements more rigorously. Since Wray *et al.* (1994) described a dependence of the *ccpA* mutant on intermediates of the TCA cycle, the effect of an addition of several intermediates to C minimal plates supplemented with glucose on growth of the mutant was tested. As shown in Table 4, glutamate was most effective in restoring growth out of the compounds tested. Acetate had no effect at all, however, it resulted also in delayed growth of the wild type and the *sgd-1* mutant QB5412. Based on these results, the *ccpA* strain QB5407 behaved formally like a glutamate auxotroph. Glutamate requirement due to a *glt* mutation affecting glutamate synthase can also be relieved by the addition of glutamine, aspartate or histidine (Bohannon *et al.*, 1985). The effect of an addition of these amino acids on growth of the *ccpA* mutant was therefore also tested. While glutamine and aspartate were as effective as or even more effective than glutamate in restoring growth, addition of histidine impaired growth under the conditions used here. In order to determine whether the bacteria metabolize glucose or glutamate on

Table 4. Effect of Different Compounds on Growth of a *ccpA* Mutant Strain^a

Compound added to C minimal agar ^c	Growth ^b		
	168 wild type	QB5407 <i>ccpA</i>	QB5412 <i>ccpA sgd-1</i>
Glc	++	-	++
Glc pyruvate	++	+/-	++
Glc acetate	+	-	+
Glc citrate	++	+/-	+
Glc α -ketoglutaric acid	++	+/-	++
Glc succinate	++	+/-	++
Glc glutamate	++	+	++
Glc glutamine	++	++	++
Glc aspartate	++	+	++
Glc histidine	+	-	-
Glc Glu 5 mM	++	+	++
Glc Glu 2 mM	++	+	++
Glc Glu 1 mM	++	+	++
Glc Glu 0,5 mM	++	+	++
Glu 5 mM	-	-	-
Glu 10 mM	-	-	-
Glu 20 mM	-	-	-
Glu 50 mM	-	-	-
Glc	++	-	++
C-N Glc	-	-	-
C-N Glc Glu	++	+	++

^a Bacteria were pregrown on SP plates and fresh colonies were streaked onto C plates containing the indicated sources of carbon and nitrogen. Plates were incubated at 37°C for two days. Abbreviations: Glc, glucose; Glu, glutamate. C-N, C minimal medium without ammonium ions).

^b ++, opaque 2- to 3-mm colonies; +, opaque 1-mm colonies; +/-, translucent pin point colonies; -, no growth at all:

^c Glucose was added at 55 mM, all other compounds at 5 mM unless otherwise stated.

plates supplemented with both compounds, growth on plates containing glutamate as a single carbon source was tested. Neither the wild type nor the mutant were able to utilize glutamate as a single carbon source in the range of concentrations tested. The observation that both glucose and glutamate are required suggests that glucose might serve as a source of carbon and energy while glutamate might be utilized as a source of nitrogen. We also tested the effect of decreasing concentrations of glutamate. As little as 0.5 mM glutamate was sufficient to restore growth of the *ccpA* mutant in the presence of glucose. It was therefore also possible that glutamate was required to regulate the synthesis of some unknown factor(s) rather than being catabolized as a nitrogen source. To distinguish between these two possibilities ammonium was omitted from the minimal medium. As expected none of the strains grew on these plates. The addition of glutamate, however, allowed growth of all three strains tested. This finding suggests that the *ccpA* mutant strain QB5407 is impaired in the utilization of ammonium as a single source of nitrogen and therefore depends on the presence of amino acids in the medium.

Effect of the *ccpA* Mutation on the Expression of the *gltAB* Operon Encoding Glutamate Synthase

As mentioned above, the *ccpA* mutant strain had a phenotype similar to that of a *glt* mutant with respect to its glutamate requirement. The fact that CcpA is a regulatory

Table 5. Effect of the *ccpA* and *sgd-1* Mutations on the Expression of a *gltA'-lacZ* Fusion in CS Medium

Added sources of carbon and nitrogen	β -Galactosidase activity ^a		
	GP16 wt	GP17 <i>ccpA</i>	GP18 <i>ccpA sgd-1</i>
none	NG ^b	NG	110
Glc (0.1%)	180	NG	150
Glc (1%)	150	NG	130
Gln	10	10	50
Gln + Glc (0.1%)	50	6	30
Gln + Glc (1%)	40	6	40

^a β -Galactosidase activity is expressed in units/mg protein.

^b NG, no growth.

protein suggested that it might affect synthesis of glutamate synthase. The expression of a *gltA'-lacZ* fusion was therefore analyzed after growth of the bacteria in CS or CSQ medium supplemented or not with glucose (glutamine was used instead of glutamate since the latter is known to repress the expression of the *gltAB* operon, (Bohannon *et al.*, 1985) (Table 5). As already described above, the *ccpA* mutant strain GP17 did not grow in CS as long as no organic source of nitrogen was added. Interestingly, the wild type strain GP16 had a requirement for glucose to grow in CS. In CSQ medium, β -galactosidase activities were low in both strains due to repression of *gltAB* expression by glutamate (Bohannon *et al.*, 1985). However, while the expression of the *gltA'-lacZ* fusion was inducible by glucose in the wild type strain this was not true for the isogenic *ccpA* mutant. This finding suggests that glucose-dependent induction of the *gltAB* operon requires the CcpA protein which might act as a positive regulator as proposed for expression of the *ack* gene encoding acetate kinase (Grundy *et al.*, 1993). The suppression of the growth deficiency in the *sgd*-mutants suggested in the light of the evidence presented above that these mutations might affect the expression of the *gltAB* operon. To test this hypothesis, β -galactosidase activities of strain GP18 (*ccpA::spc sgd-1*) were determined as described above and compared to those of the isogenic wild type strains (Table 5). Indeed, the *sgd-1* mutation restored expression of the *gltA'-lacZ* fusion in a *ccpA* mutant background. This expression is constitutive irrespective of the presence of glucose in the growth medium. The *sgd-1* mutation confers the bacteria also with the ability to grow on CS medium.

The experiments described above did not resolve whether lack of *gltAB* operon expression was the single reason for the growth defect of the *ccpA* mutant. To address this question we placed the *gltAB* operon under the control of the *spac* promoter that is inducible by IPTG. Growth of strains 168, QB5407 (*ccpA*), GP123 (*gltAB* under control of p_{spac}), and GP124 (*ccpA gltAB* under control of p_{spac}) on C plates supplemented with glucose was tested in the presence and absence of IPTG. While the *ccpA*⁺ strains grew on both media, no growth was observed for the *ccpA* mutants irrespective of the expression of the *gltAB* operon. This result suggests that factors involved in ammonium assimilation different from *gltAB* might be subject to positive control by CcpA (see discussion).

Discussion

The CcpA protein is central to the regulation of carbon metabolism in Gram-positive bacteria. Mutations in *ccpA* pleiotropically result in the loss of carbon catabolite repression of numerous catabolic genes and operons and in the loss of glucose activation of genes involved in the excretion of excess carbon (Henkin, 1996). In order to regulate transcription of the controlled genes, CcpA needs to bind a cofactor which may be either glucose-6-phosphate, NADP, HPr or Crh phosphorylated at serine-46 (Deutscher *et al.*, 1995; Galinier *et al.*, 1997; Gösseinger *et al.*, 1997; Jones *et al.*, 1997; Miwa *et al.*, 1997; Kim *et al.*, 1998). The generation of the cofactors and their subsequent interaction with CcpA link the metabolic status of the cell to the activity of the regulator. Thus, the function of CcpA in catabolite repression in *B. subtilis* is equivalent to that of the cAMP receptor protein Crp in *Escherichia coli* and other enteric bacteria.

In addition to a loss of carbon catabolite repression *ccpA* mutants exhibit a second phenotype, *i.e.* they grow poorly on minimal media. Since other mutants pleiotropically defective in catabolite repression (such as the *ptsH1* mutant which carries the *ptsH*-S46A mutation) grew normally on minimal media we concluded that CcpA might have functions that had not yet been elucidated. The severe growth defect of the *ccpA* mutant led us to ask whether loss of catabolite repression was really an intrinsic feature conferred by the *ccpA* mutation or simply the result of inefficient utilization of glucose and other repressing carbon sources. Data presented here clearly demonstrate that *ccpA* mutants are not affected in the transport of glucose. The genes of the *B. subtilis ptsGHI* operon are normally expressed in a *ccpA* mutant. Moreover, the activity of the components of the glucose-specific PTS is not affected by the *ccpA* mutation. These findings are in accordance with previous data which show that several sugar-specific permeases do not depend on a functional CcpA to be expressed and active (Deutscher *et al.*, 1994; Martin-Verstraete *et al.*, 1995; Krüger *et al.*, 1996). In addition, growth with different carbon sources is affected irrespective of the system involved in transport of the given carbohydrate. Thus, we conclude that factors different from carbohydrate transport cause the growth defect of the *ccpA* mutant. It was suggested that high glycolytic activity is required to evoke carbon catabolite repression in *B. subtilis* (Nihashi and Fujita, 1984; Krüger *et al.*, 1993; Deutscher *et al.*, 1994). The evidence presented here suggests that glycolytic activity is, however, not the factor causing the *ccpA* phenotypes. The growth deficiency does not depend on the pathway used for the catabolism of a carbon source. In addition, compelling evidence has recently been presented demonstrating the interaction between the elements involved in catabolite repression (Deutscher *et al.*, 1995; Fujita *et al.*, 1995; Gösseinger *et al.*, 1997; Jones *et al.*, 1997; Miwa *et al.*, 1997; Kim *et al.*, 1998). These data prove the direct role of CcpA and its cofactors in DNA binding and subsequent catabolite repression.

To further our understanding of the role of CcpA in cellular physiology we isolated suppressor mutants that were able to grow on minimal medium. The corresponding mutations did, however, not restore carbon catabolite repression suggesting that the phenotypes of the *ccpA* mutant were not related to each other. This notion is

supported by the finding that *abrB* overexpression due to *spo0A* mutation restored growth of the *ccpA* mutant but did not restore catabolite repression of the *B. subtilis* ribose transport operon (Strauch, 1995).

A detailed analysis of the growth requirements of the *ccpA* mutant revealed that the mutant was auxotrophic for glutamate. The same phenotype is conferred by mutations in the *gltA* or *gltB* genes encoding the subunits of glutamate synthase, a key enzyme of ammonium assimilation in *B. subtilis*. Glutamate synthase catalyzes the reductive amination of α -ketoglutaric acid. Expression of the *gltAB* operon is controlled by the nitrogen source and mediated by the transcriptional activator GltC (Bohannon *et al.*, 1985; Bohannon and Sonenshein, 1989). Here, we demonstrate that *gltAB* operon expression is induced by glucose and that this induction depends on a functional *ccpA* gene. These findings suggest that the glutamate auxotrophy observed for the *ccpA* mutant might be related to the lack of *gltAB* operon expression. This notion is reinforced by the analysis of the *ccpA sgd-1* double mutant which is capable of growing on a minimal medium with glucose and ammonium as sole carbon and nitrogen sources, respectively. The *sgd-1* mutation renders the expression of the *gltAB* operon independent from the presence of glucose in the medium and from the *ccpA* gene product. The finding that inducible expression of the *gltAB* operon does not restore the growth defect of the *ccpA* mutant suggests that (an) other factor(s) positively controlled by CcpA might be required for ammonium assimilation. It is not known how ammonium transport is regulated in *B. subtilis*, but it is conceivable that the gene(s) encoding the ammonium transporter are coordinately regulated with the *gltAB* operon and thus under positive control of CcpA. In addition, glutamine was even more effective in supporting growth of the *ccpA* mutant, this might, however be due to more efficient utilization of glutamate as a source of nitrogen. The presence of a potential *cre* sequence upstream of the promoter of the *gltP* gene encoding the glutamate transporter might suggest positive regulation of glutamate transport by CcpA which is missing in the *ccpA* mutant (Tolner *et al.*, 1995).

It has long been proposed that there is a link between carbon and nitrogen metabolism. Recently, the PTS was suggested and shown to connect both processes in *E. coli* and other Gram-negative bacteria (Powell *et al.*, 1995). In these bacteria, the *rpoN* gene encodes an alternative sigma factor involved in the expression of genes of nitrogen assimilation. Interestingly, the *rpoN* operon contains genes, the products of which resemble PTS proteins. One of these proteins, EIIA^{Ntr} was found to be required for an efficient utilization of organic sources of nitrogen in the presence of multiple carbon sources. Moreover, also non-metabolizable PTS substrates such as methyl- α -glucoside inhibit the utilization of organic nitrogen sources in an *E. coli ptsN* mutant which does not express a functional EIIA^{Ntr} (Powell *et al.*, 1995). Thus, PTS components link carbon and nitrogen metabolism in *E. coli*. It is well established that similar regulatory circuits such as carbon catabolite repression are often achieved by different mechanisms in Gram-negative and Gram-positive bacteria (for a review see Hueck and Hillen, 1995). In Bacilli, the CcpA protein, the central component of the carbon catabolite repression regulatory system, is also engaged in the control of nitrogen assimilation. The data presented here clearly establish that

Table 6. *B. subtilis* Strains used in this Work. Arrows Indicate Construction by Transformation

Strain	Genotype or description	Source or reference
168	<i>trpC2</i>	Laboratory stock
BGW2	<i>trpC2 lys-3 ccpA::Tn917</i>	Krüger <i>et al.</i> , 1993
BGW404	<i>trpC2 lys-3 amyE::(bglPΔ11'-lacZ cat)</i>	Krüger <i>et al.</i> , 1996
GM1225	<i>trpC2 pheA1 ΔbgaX amyE::(gntRK'-lacZ) ccpA::Tn917 spc^a</i>	Deutscher <i>et al.</i> , 1994
IS58	<i>trpC2 lys-3</i>	Smith <i>et al.</i> , 1980
LG219	<i>amyE::(cat Φ(gltC'-gusA) Φ(gltAp19-lacZ)</i>	B. Belitsky
QB5081	<i>trpC2 amyE::(levD'-lacZ cat)</i>	Martin-Verstraete <i>et al.</i> , 1992
QB5407	<i>trpC2 ccpA::Tn917 spc</i>	GM1225 → 168
QB5412	<i>trpC2 ccpA::Tn917 spc sgd-1</i>	see Results
QB5413	<i>trpC2 ccpA::Tn917 spc sgd-2</i>	see Results
QB5414	<i>trpC2 ccpA::Tn917 spc sgd-3</i>	see Results
QB5415	<i>trpC2 ccpA::Tn917 spc sgd-5</i>	see Results
QB5418	<i>trpC2 amyE::(ptsHI'-lacZ cat)</i>	pTS105 → 168
QB5448	<i>trpC2 amyE::(ΔLA ptsG'-lacZ aphA3)</i>	Stülke <i>et al.</i> , 1997
GP1	<i>trpC2 amyE::(ptsH'-lacZ cat) ccpA::Tn917 spc</i>	QB5407 → QB5418
GP10	<i>trpC2 amyE::(ΔLA ptsG'-lacZ aphA3) ccpA::Tn917 spc</i>	QB5407 → QB5448
GP16	<i>trpC2 amyE::(cat Φ(gltC-gusA) Φ(gltAp19-lacZ))</i>	LG219 → 168
GP17	<i>trpC2 amyE::(cat Φ(gltC-gusA) Φ(gltAp19-lacZ)) ccpA::Tn917(Δerm lacZ)::spc</i>	LG219 → QB5407
GP18	<i>trpC2 amyE::(cat Φ(gltC-gusA) Φ(gltAp19-lacZ)) ccpA::Tn917(Δerm lacZ)::spc sgd-1</i>	LG219 → QB5412
GP25	<i>trpC2 amyE::(bglPΔ11'-lacZ cat)</i>	BGW404 → 168
GP26	<i>trpC2 ccpA::Tn917 spc amyE::(bglPΔ11'-lacZ cat)</i>	GP25 → QB5407
GP27	<i>trpC2 ccpA::Tn917 spc sgd-1 amyE::(bglPΔ11'-lacZ cat)</i>	GP25 → QB5412
GP28	<i>trpC2 ccpA::Tn917 spc amyE::(levD'-lacZ cat)</i>	QB5081 → QB5407
GP29	<i>trpC2 ccpA::Tn917 spc sgd-1 amyE::(levD'-lacZ cat)</i>	QB5081 → QB5412
GP123	<i>trpC2 gltA::pGP121</i>	pGP121 → 168
GP124	<i>trpC2 ccpA::Tn917 spc gltA::pGP121</i>	pGP121 → QB5407

^a For clarity, the genotype of strain GM1225 was renamed *ccpA::Tn917 spc* as proposed earlier (Martin-Verstraete *et al.*, 1995).

a functional *ccpA* gene product is necessary for the expression of the operon encoding glutamate synthase. A *cre* sequence could, however, not be identified in the *gltAB* upstream region suggesting that some factor(s) controlled by CcpA are involved in the regulation identified here. In contrast to the situation in *E. coli*, glutamine is the preferred source of nitrogen in *B. subtilis* rather than ammonium (Fisher and Sonenshein, 1991). Moreover, the presence of glutamine in the medium results in nitrogen catabolite repression of the utilization of secondary sources of nitrogen such as arginine (Fisher and Sonenshein, 1991). The genes specified by the *sgd* mutations are interesting candidates for providing a direct link between carbon and nitrogen metabolism in addition to CcpA. The identification of these genes is therefore of crucial importance for our better understanding of how the metabolic routes are interrelated.

Recent findings suggest that CcpA might not only be involved in the regulation of carbon and nitrogen metabolism but also in anaerobic fermentative growth. Acetate and butandiol have recently been shown to be fermentation products of *B. subtilis* when grown anaerobically in the presence of glucose and pyruvate (Nakano *et al.*, 1997). Both fermentative pathways require activation by CcpA as demonstrated for expression of the *ackA* gene encoding acetate kinase and acetoin production (Henkin *et al.*, 1991; Grundy *et al.*, 1993). In fact, a *ccpA* mutation was first isolated as *alsA* allele involved in acetoin production (Zahler *et al.*, 1976). Thus, the CcpA protein seems to be a central regulator of carbon, energy and nitrogen metabolism in *B. subtilis* and probably in other Gram-positive bacteria.

The relationship between carbon and nitrogen metabolism in *B. subtilis* described here provokes a lot of open questions for future research. It will be interesting to

analyze the signals which result in the involvement of CcpA in the regulation of ammonium assimilation and the molecular mechanism by which CcpA interferes with the expression of the *gltAB* operon.

Experimental Procedures

Bacterial Strains, Plasmids and Culture Media

The *Bacillus subtilis* strains used in this work are listed in Table 6. Plasmid pTS105 (Gonzy-Tréboul *et al.*, 1989) was used to introduce a *ptsHI-lacZ* fusion into the *B. subtilis* chromosome. *E. coli* DH5α (Sambrook *et al.*, 1989) was used for cloning experiments.

B. subtilis was grown in ASM minimal medium (Stülke *et al.*, 1993), SP medium, C minimal medium (Martin-Verstraete *et al.*, 1990) or in modified C medium without a source of nitrogen (C-N; 70 mM K₂HPO₄·3 H₂O, 30 mM KH₂PO₄, 25 mM Na₂SO₄, 0.5 mM MgSO₄, 10 μM MnSO₄, 22 mg ferric ammonium citrate/l). The media were supplemented with auxotrophic requirements (at 50 mg/l) and carbon sources. CS is C medium supplemented with potassium succinate (6 g/l). CSE, CSG and CSQ media are CS supplemented with potassium glutamate (8 g/l), glucose (1 g/l), and glutamine (1.5 g/l), respectively. C Glc is C medium supplemented with glucose (4 g/l).

E. coli was grown in LB medium and transformants were selected on plates containing ampicillin (100 μg/ml).

LB, SP and C plates were prepared by the addition of 17 g Bacto agar/l (Difco) to LB, SP or C medium, respectively.

DNA Manipulation and Plasmid Construction

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the Nucleotrap Gel Extraction kit (Macherey and Nagel, Düren, Germany). *Pfu* DNA polymerase was used for the polymerase chain reaction as recommended by the manufacturer. DNA sequences were determined using the dideoxy chain termination method (Sambrook *et al.*, 1989).

To place the *gltAB* operon under the control of an inducible promoter, plasmid pGP121 was constructed as follows. Integration plasmid pMUTIN2 (S.D. Ehrlich) allows (i) selection of erythromycin resistance in *B. subtilis*, and (ii) placement of genes downstream of P_{spac} which allows induction of transcription in the presence of isopropyl-β-D-thiogalactosylpyranoside (IPTG). A 915 bp DNA fragment containing the upstream part of *gltAB* was

amplified by PCR using the primers SB32 (5' ATAGAATTCCCGGTATTATCGGAAATTGATCGGGG) and SB33 (5' ATACCGCGGAAGAGTCACTGCCATCAGCATTGAG). The PCR product was digested with *EcoRI* and *SacI* (the sites were introduced upon PCR, they are underlined in the primer sequences) and cloned into pMUTIN2 cut with the same enzymes.

Transformation and Characterization of Phenotype

B. subtilis was transformed with chromosomal DNA according to the two-step protocol described previously (Kunst and Rapoport, 1995). Transformants were selected on SP plates containing spectinomycin (Spc 100 µg/ml), chloramphenicol (Cm 5 µg/ml), kanamycin (Km 5 µg/ml) or erythromycin plus lincomycin (Em 1 µg/ml and Lin 25 µg/ml).

Doubling times were determined by growing the bacteria in modified ASM medium (Krüger *et al.*, 1993) or in C minimal medium supplemented with 1% (w/v) of the carbon source. ASM or CSE were used for the precultures.

Quantitative studies of *lacZ* expression and β-xylosidase synthesis in *B. subtilis* in liquid medium were performed as follows: cells were grown in CSE medium supplemented with the carbon sources indicated. Xylose (1 g/l) was used as inducer for β-xylosidase. Cells were harvested at OD₆₀₀ 0.6 to 0.8 for cultures in CSE medium and 0.8 to 1 for cultures in CSE medium with sugar. Cell extracts were obtained by treatment with lysozyme and DNase. β-Galactosidase and β-xylosidase activities were determined as previously described using o-nitrophenyl-galactoside and p-nitrophenyl-xyloside, respectively, as substrate (Miller, 1972; Lindner *et al.*, 1994). One unit is defined as the amount of enzyme which produces 1 nmol of o-nitrophenol (β-galactosidase) or p-nitrophenol (β-xylosidase) per min at 28 °C.

Uptake of Radioactive Glucose in vivo

B. subtilis strains were grown in CSE medium with glucose (10 g/l). Sugar uptake assays were performed as described previously (Stülke *et al.*, 1997). Medium C supplemented with 10 g/l glycerol was used for incorporation of the isotope at 37°C. Exponentially growing cells were harvested at an OD₆₀₀ of 0.6 to 0.7 and washed once with the incorporation medium. Labelled [¹⁴C] D-glucose (184 mCi mmol⁻¹) and non-labelled glucose (final concentration 0.4 mM) were added. Samples were taken and treated as described previously (Martin-Verstraete *et al.*, 1990).

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